and complete mixing of the liquids. Shake the flasks intermittently for 1 hour. Proceed as directed in paragraph (e) of this section.

(2) *Finished product solutions.* Prepare the sample for assay as directed in the individual section for each antibiotic product to be tested.

(e) *Procedure.* Inject 2.5 microliters of each solution into the gas chromatograph. Use the conditions and materials listed in paragraphs (a), (b), and (c) of this section. The conditions should be adequate to maintain a stable base line and provide at least 60 percent deflection of the recorder scale by the spectinomycin peak. The resolution of the peaks should be complete. The internal standard will be eluted before spectinomycin. Calculate the spectinomycin content as directed in paragraph (f) of this section.

(f) *Calculations.* Calculate the spectinomycin content of the sample as follows:

$$\text{Micrograms of spectinomycin per milligram} = \frac{R_u \times W_s \times f}{R_s \times W_u}$$

where:

R_u=Area of spectinomycin sample peak (at a retention time equal to that observed for the spectinomycin standard)/Area of internal standard peak;

R_s=Area of the spectinomycin standard peak/Area of internal standard peak;

W_s=Weight of the spectinomycin working standard in milligrams;

W_u=Weight of the sample in milligrams;

f=Potency of the spectinomycin working standard in micrograms per milligram.

§ 436.308 Paper chromatography identity test for tetracyclines.

(a) *Equipment*—(1) *Sheet* (*chromatographic*). Whatman No. 1 filter paper for chromatography, 20 × 20 centimeters.

(2) *Chamber* (*chromatographic*). Cylindrical glass chromatographic jar, 25 centimeters high by 12 centimeters in diameter, with a ground-glass lid.

(3) *Preparation of solutions*—(i) *pH3.5* buffer. Mix 13.93 volumes of 0.1*M* citric acid with 6.07 volumes of 0.2*M* of disodium phosphate.

(ii) *Solvent* (*organic phase*). Mix chloroform, nitromethane, and pyridine in volumetric proportions of 10:20:3, respectively.

(b) *Preparation of spotting solutions.* Prepare solutions of the working standard and sample as follows: Accurately weigh a portion of the working standard and sample and dilute with methanol to obtain a concentration of 1 milligram per milliliter of antibiotic to be tested.

(c) *Procedure.* Fill the chamber to a depth of 0.6 centimeter with freshly prepared solvent. Draw a starting line about 2.5 centimeters from and parallel to the bottom of the sheet. Wet the sheet thoroughly with the pH 3.5 buffer and blot it firmly between sheets of absorbent paper. Starting about 5 centimeters from the edge of the sheet and at 1.5-centimeter intervals, apply to the starting line 2 microliters each of standard solution, sample solution, and a 1:1 mixture of the standard and sample solutions. Allow a few minutes for the sheet to dry partially, and while still damp place it in the chamber with the bottom edge touching the solvent. When the solvent front has risen about 10 centimeters, remove the sheet from the chamber. Expose the paper to ammonia vapor. Examine the dried sheet under a strong source of ultraviolet light and record the position of any fluorescent spots. Measure the distance the solvent front traveled from the starting line and the distance that the fluorescent spots are from the starting line. Calculate the R_f value by dividing the latter by the former.

[39 FR 18944, May 20, 1974, as amended at 44 FR 30333, May 5, 1979; 45 FR 16472, 16474, Mar. 14, 1980]

§ 436.309 Anhydrotetracyclines and 4-epianhydrotetracycline.

Determination of 4-epianhydrotetracycline and anhydrotetracyclines in tetracycline, tetracycline hydrochloride, tetracycline phosphate, and in dosage forms thereof is as follows:

(a) *Screening procedure for total anhydrotetracyclines content*—(1) *Sample solution preparation*—(i) *Bulk packaged for repacking or for use in the manufacture of another drug.* Accurately weigh approximately 50 milligrams of the sample into a 50-milliliter volumetric flask and add 10 milliliters of 0.1*N* hydrochloric acid. Shake until sample is

completely dissolved, and then dilute to volume with water.

(ii) *Sterile dispensing containers.* Proceed as directed in paragraph (a)(1)(i) of this section.

(iii) *Capsules.* Transfer a representative quantity of capsule contents equivalent to 250 milligrams of tetracycline hydrochloride to a 250-milliliter volumetric flask. Add 50 milliliters of 0.1*N* hydrochloric acid and shake on a mechanical shaker for 5 minutes. Dilute to volume with water and filter through a fluted filter paper. Discard the first 20 milliliters of filtrate and collect the next 20 milliliters.

(iv) *Tablets.* Grind a representative number of tablets to a fine powder. Transfer an amount of the powder equivalent to 250 milligrams of tetracycline hydrochloride to a 250-milliliter volumetric flask. Add 50 milliliters of 0.1*N* hydrochloric acid and shake on a mechanical shaker for 5 minutes. Dilute to volume with water and filter through a fluted filter paper. Discard the first 20 milliliters of filtrate and collect the next 20 milliliters.

(v) *Oral powders and suspensions.* Proceed as described in paragraph (b) of this section.

(2) *Test procedure.* Using a suitable spectrophotometer, determine the absorbance of the sample solution prepared as directed in paragraph (a)(1) of this section at 430 millimicrons using 0.02*N* hydrochloric acid as a blank. Then accurately dilute 1.0 milliliter of the sample solution to 100 milliliters with 0.02*N* hydrochloric acid and determine the absorbance of this solution at 356 millimicrons, using 0.02*N* hydrochloric acid as a blank.

(3) *Calculations.*

$$\text{Percent anhydrotetracyclines} = \frac{[a430 - (a356 \times 0.0019)] \times 100}{195}$$

where:

*a*430=Absorptivity (1%, 1 cm.) of sample at 430 millimicrons;

$$\text{For bulk, absorptivity} = \frac{\text{Absorbance} \times 50 \times 10}{\text{Milligrams of sample}}$$

For sterile dispensing containers, capsules, and tablets; absorptivity=Absorbance×10; *a*356=Absorptivity (1%, 1 cm.) of sample at 356 millimicrons;

$$\text{For bulk, absorptivity} = \frac{\text{Absorbance} \times 50 \times 1000}{\text{Milligrams of sample}}$$

For sterile dispensing containers, capsules and tablets; absorptivity=Absorbance×1,000; 0.0019×Absorbance ratio (*A*430/*A*356) observed with tetracycline;

195=Absorptivity (1%, 1 cm.) of anhydrotetracycline hydrochloride at 430 millimicrons.

(4) *Evaluation.* If the total anhydrotetracyclines content determined by the screening procedure described in paragraph (a) of this section exceeds 2 percent for bulks and 3 percent for injectables, tablets, and capsules, perform the determination for anhydrotetracyclines and 4-epianhydrotetracycline described in paragraph (b) of this section. If the results of the test described in paragraph (a) of this section for total anhydrotetracyclines content are within the required limits in the case of bulks, injectables, tablets, and capsules, these results may be submitted in lieu of the results of the test for 4-epianhydrotetracycline and that test as described in paragraph (b) of this section need not be performed.

(b) *Determination of anhydrotetracyclines content and 4-epianhydrotetra-cycline content*—(1) *Apparatus and reagents*—(i) Chromatographic tubes (15 millimeters ID × 170 millimeters long having an outlet tube 4 millimeters ID × 50 millimeters long).

(ii) pH meter standardized at pH 7.0 and at pH 10.0.

(iii) Diatomaceous earth, acid-washed (Celite 545 or equivalent).

(iv) EDTA buffer. Dissolve 0.1 mole ethylenediaminetetraacetic acid disodium salt in 800 milliliters of water.

Adjust to pH 7.8 with ammonium hydroxide, reagent grade, and dilute to 1 liter with water.

(v) Chloroform, spectrophotometric grade.

(vi) Diluted ammonium hydroxide: Mix 1 volume of ammonium hydroxide, reagent grade, with 9 volumes of distilled water.

(vii) 0.1*N* hydrochloric acid.

(viii) 1.0*N* hydrochloric acid.

(2) *Preparation of support phase.* Add 5 milliliters of EDTA buffer to 10 grams of diatomaceous earth and mix until the diatomaceous earth is uniformly moistened. It will no longer be free-flowing.

(3) *Preparation of sample solutions.* Prepare the sample solutions as follows:

(i) *Tetracycline, tetracycline phosphate complex, and tetracycline hydrochloride bulk packaged for repacking or for use in the manufacture of another drug.* Place an amount of sample equivalent to 250 milligrams of tetracycline hydrochloride into a 50-milliliter beaker and dissolve in 10 milliliters of 0.1*N* hydrochloric acid. Immediately adjust the pH to 7.8 with the diluted ammonium hydroxide, and if necessary, with 1.0*N* hydrochloric acid and 0.1*N* hydrochloric acid. Quantitatively transfer this solution to a 50-milliliter volumetric flask by rinsing the beaker with EDTA buffer, fill to volume with EDTA buffer and shake well. Use this solution without delay to prepare a column as directed in paragraph (b)(4) of this section.

(ii) *Capsules.* Proceed as directed in paragraph (b)(3)(i) of this section, except pool the contents of a representative number of capsules and use an amount of the pooled capsule contents equivalent to 250 milligrams of tetracycline hydrochloride.

(iii) *Tablets.* Proceed as directed in paragraph (b)(3)(i) of this section, except grind tablets to a powder in a small mortar and use an amount of powder equivalent to 250 milligrams of tetracycline hydrochloride.

(iv) *Oral suspension and pediatric drops.* Place 5 milliliters of oral suspension equivalent to 125 milligrams of tetracycline hydrochloride or 2 milliliters of pediatric drops equivalent to 200 milligrams of tetracycline hydrochloride into a 50-milliliter beaker and add sufficient 0.1*N* hydrochloric acid to make 10 milliliters. Quickly adjust the pH to 7.8 with the diluted ammonium hydroxide, and if necessary, with 1*N* hydrochloric acid and 0.1*N* hydrochloric acid. Quantitatively transfer this solution to a 25-milliliter flask by rinsing the beaker with EDTA buffer, fill to volume with EDTA buffer, and shake well. Use this solution without delay to prepare a column as directed in paragraph (b)(4) of this section.

(v) *Oral powders.* Reconstitute as directed in the labeling and proceed as directed in paragraph (b)(3)(iv) of this section.

(vi) *Sterile dispensing containers.* Proceed as directed in paragraph (b)(3)(i) of this section.

(4) *Column preparation.* Pack support phase into the chromatographic tube by increments and firmly tamp down each increment. Do not use any glass wool in the column outlet. Add enough support phase to the column to reach a height of 9 to 11 centimeters; then add 1 milliliter of sample solution to 1 gram of diatomaceous earth in a small beaker, and mix thoroughly. Pack the sample: diatomaceous earth mixture on top of the column. Dry wash the beaker with support phase and pack an additional 1-centimeter layer of support phase on top of the sample layer.

(5) *Column elution and fraction collection.* Within 30 minutes after preparing the column, elute with chloroform. Collect 5 successive fractions of 5 milliliters, 5 milliliters, 10 milliliters, 10 milliliters, and 5 milliliters. During elution, two clear separate yellow bands will appear on the column. The first band is anhydrotetracyclines and will almost always elute in the first 5-milliliter fraction, but occasionally in the first and second 5-milliliter fractions. The second band is 4-epianhydrotetracycline and will elute in the remaining fractions. Label the fraction or fractions containing the first yellow band anhydrotetracyclines. Label the fractions after the first yellow band 4-epianhydrotetracycline. Determine the absorbance of each fraction at a wavelength of 438 nanometers using a suitable spectrophotometer equipped with a 1.0-centimeter cell and chloroform as the blank. If necessary,

make appropriate dilutions with choloroform to obtain a readable value.

(6) *Calculations*—(i) *Percent anhydrotetracyclines.* Calculate the percent anhydrotetracyclines as follows:

$$\text{Number of milligrams of anhydrotetracyclines in each fraction containing anhydrotetracyclines} = \frac{A \times b \times c}{20.28}$$

where:

A=Absorbance of the sample solution at 438 nanometers;

b=Volume of fraction in milliliters;

c=Dilution factor of the fraction (for example, if 2 milliliters of the fraction are diluted to 10 milliliters for reading, *c* will be 5).

20.28=Absorptivity (1 milligram per milliliter, 1 centimeter) of anhydrotetracyclines in chloroform at 438 nanometers.

Total weight of anhydrotetracyclines in the sample=Sum of weights of anhydrotetracyclines in the fractions labeled anhydrotetracyclines × Number of milliliters in the sample solution

Percent anhydrotetracyclines in tetracycline, tetracycline hydrochloride, tetracycline phosphate complex bulk packaged for repacking or for use in the manufacture of another drug=

$$100 \times \frac{\text{Total weight of anhydrotetracyclines in the sample}}{\text{Weight of the sample}}$$

Percent anhydrotetracyclines in dosage forms=

$$100 \times \frac{\text{Total weight of anhydrotetracyclines in the sample}}{\text{Tetracycline content of the sample}}$$

(ii) *Percent 4-epianhydrotetracycline.* Calculate the percent 4-epianhydrotetracycline as follows:

$$\text{Number of milligrams of 4-epianhydrotetracycline in each fraction labeled 4-epianhydrotetracycline} = \frac{A \times b \times c}{20.08}$$

where:

A=Absorbance of the sample solution at 438 nanometers;

b=Volume of the fraction in milliliters;

c=Dilution factor of the fraction (for example, if 2 milliliters of the fraction are diluted to 10 milliliters for reading, *c* will be 5);

20.08=Absorptivity (1 milligram per milliliter, 1 centimeter) of 4-epianhydrotetracycline in chloroform at 438 nanometers.

Total weight of 4-epianhydrotetracycline in the sample=Sum of weights of 4-epianhydrotetracycline in the fractions labeled 4-epianhydrotetracycline × Number of milliliters in the sample solution

Percent 4-epianhydrotetracycline in tetracycline, tetracycline hydrochloride, tetracycline phosphate complex bulk packaged for repacking or for use in the manufacture of another drug=

$$100 \times \frac{\text{Total weight of 4-epianhydrotetracycline in the sample}}{\text{Weight of the sample}}$$

Percent 4-epianhydrotetracycline in dosage forms=

$$100 \times \frac{\text{Total weight of 4-epianhydrotetracycline in the sample}}{\text{Tetracycline content of the sample}}$$

[39 FR 18944, May 30, 1974, as amended at 40 FR 22251, May 22, 1975; 43 FR 11153, Mar. 17, 1978]

§ 436.310 Thin layer chromatography identity test for mitomycin.

(a) *Equipment*—(1) *Chromatography tank.* A rectangular tank, approximately 9 × 9 × 3.5 inches, lined with filter paper and with a solvent trough on the bottom.